Effects of resveratrol on cancer cachexia in a mouse model

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Abstract

Cancer cachexia, characterized by muscle wasting and fatigue, affects many cancer patients. Inflammatory processes are implicated in the pathobiology of cancer cachexia. Resveratrol, a compound found in red grapes, peanuts, and dark chocolate has been found to exhibit anti-inflammatory properties. This study evaluated resveratrol as an intervention to decrease or reverse symptoms of muscle wasting and fatigue in tumor bearing mice. In mice, fatigue is measured as decreased voluntary wheel running activity (VWRA). The sample included 4 groups of seven C57BI/6 female mice. Using a quantitative, experimental design, each mouse was housed alone and acclimated for 10 days to a running wheel. Half the mice were injected with Lewis Lung Carcinoma cells and half served as healthy controls. Half the tumor and control mice were treated with a slow release pellet planted under the skin designed to deliver 1 mg/kg/day of resveratrol over 21 days, and half received a placebo pellet. Wheel running activity was measured at day 0, 7, 14, and 19 of tumor growth. At the end of the experiment, gastrocnemius muscles were removed and weighed to evaluate muscle wasting. A two-way (tumor drug) analysis of variance was used to determine the effect of tumor growth and resveratrol on muscle mass and fatigue. Analysis showed no effects of resveratrol on muscle mass, VWRA, or tumor growth compared to control animals. However, VWRA was significantly correlated with muscle mass. The data indicate that resveratrol at this dose had no effect on muscle wasting or fatigue in tumor-bearing mice. However, loss of muscle mass may directly contribute to fatigue as indicated by the correlation between muscle mass and VWRA. More research is

needed to develop effective interventions to reduce muscle wasting, which may reduce fatigue in patients with cancer cachexia.

Introduction

Cancer cachexia is a syndrome of progressive weight loss characterized by the wasting of body fat and lean muscle, along with fatigue, weakness, and decreased appetite (Hemming & Maher, 2005). Both muscle wasting and fatigue are prevalent symptoms experienced by patients. Cancer patients who suffer from cancer cachexia have higher incidences of morbidity and mortality, decreased response to treatment, and more adverse effects from cancer treatment (Skipworth, Stewart, Dejong, Preston, & Fearon, 2007). The cause of cancer cachexia is still unknown. Thus treatments to decrease or reverse cachexia have yet to be found.

One particular process that may contribute to cancer cachexia is the inflammatory response. Tumors induce pro-inflammatory cytokines that activate catabolic processes. These cytokines directly activate the ubiquitin-proteosome pathway, which causes myosin degradation, muscle wasting, and decreased muscle mass and strength (Skipworth, et al., 2007; Deans, Wigmore, Paterson-Brown, Black, Ross, & Fearon, 2005; Cai, *et al.,* 2004). Resveratrol, a chemically simple compound found in red grapes, peanuts, and dark chocolate has been found to exhibit anti-tumor and anti-inflammatory effects (Udenigwe, Ramprasath, Aluko, & Jones, 2008). Resveratrol may reduce the release of inflammatory cytokines, leading to decreased muscle wasting and fatigue.

The purpose of this study is to evaluate the effects of resveratrol on muscle wasting and fatigue in a mouse model of cancer cachexia. In mice, fatigue is modeled as a decrease in voluntary wheel running activity (VWRA). Gastrocnemius muscle mass will be used as a measure of muscle wasting.

This study is important because cancer is one of the leading causes of death in the United States (Centers for Disease Control and Prevention (CDC), 2007), and approximately half of all cancer patients experience cancer cachexia during the course of their disease. Futhermore, because cancer survival rates are rising, many more people are living with weakness, fatigue, and decreased muscle mass than in previous years. Cachexia is associated with increased morbidity and mortality (Hemming & Maher, 2005). This progressive syndrome affects both the psychological and physical well-being of patients, ultimately decreasing their quality of life. Thus, it is crucial to search for interventions that slow down or counteract this wasting process.

Review of Literature

Cancer cachexia is a progressive syndrome of fatigue, skeletal muscle wasting, weight loss, anemia, and anorexia (Argiles, Lopez-Soriano & Busquets, 2007). In the United States, cancer is the second leading cause of death, with 562,875 people dying from cancer in 2007 (American Cancer Society, 2010). According to the American Cancer Society, there are over 11 million Americans alive today with a history of cancer (2009). Cancer cachexia affects approximately 50% of these cancer patients during their treatment, and is present in close to 100% of cancer patients who die from their disease (Norton, Peacock, & Morrison, 1987). As this syndrome progresses, it becomes both a physical and psychological burden on the patient, contributing to adverse outcomes. Patients have reported decreased quality of life, decreased response to treatment, increased treatment side effects, decreased physical performance, and increased mortality rates (Skipworth, Stewart, Dejong, Prestion & Fearon, 2007). While

many different effects of tumor growth are thought to contribute to cachexia, a growing body of evidence suggests that activation of the inflammatory system has a major influence on the progression of cachexia.

The pathobiology of cancer cachexia is not fully understood. However, studies have shown cancer cachexia is associated with a tumor-induced activation of pro-inflammatory cytokines which leads to decreased muscle mass and strength. Pro-inflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF-α) activate the ubiquitin proteolytic pathway, which causes myofibrillar protein degradation and muscle wasting (Argiles, Busquets, & Lopez-Soriano, 2006). Animals given supplements of these cytokines demonstrate a characteristic "sickness behavior," which includes decreased activity and food intake, and altered sleep patterns (Wang, 2008). TNF-α is associated with the activation of NF-kB, which is a transcription factor that increases synthesis and activity of the ubiquitin-proteosome pathway. In muscle, this is associated with increased expression of E3 ubiquitin ligases, muscle atrophy F box (MAFbx) and muscle RING finger 1 (MuRF1). These ligases selectively polyubiquininate myosin proteins to be degradated (Dodson, Baracos, Jatoi, Evans, Cella, Dalton, & Steiner, 2011). Expression of MAFbx and MuRF1 is increased in animal models of muscle wasting (Dodson et. al, 2011). Additionally, studies have found elevated levels of pro-inflammatory biomarkers in cancer patients (Wang, 2008). Thus, increased cytokine levels are associated with muscle degradation and fatigue in cachexia patients.

Evidence from animal models of cancer cachexia suggests that down regulation of the inflammatory response may decrease or reverse muscle wasting and fatigue in

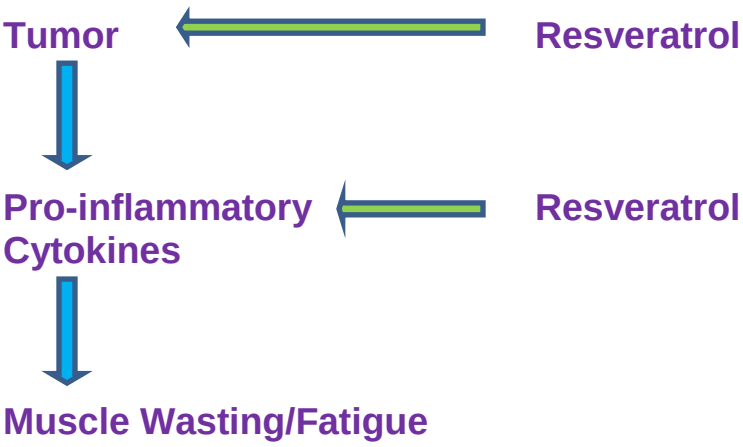
tumor-bearing animals. Previous studies indicate that non-steroidal anti-inflammatory drugs (NSAIDs) may preserve muscle mass and body weight in tumor-bearing mice (Mantovani & Madeddu, 2008). This evidence leads us to believe that other substances that suppress the inflammatory response may also decrease cachexia.

Resveratrol is a compound found in red grapes, dark chocolate, red wine, and peanuts, among others, that exhibits anticancer, antioxidant, and anti-inflammatory properties (Udenigwe, Ramprasath, Aluko, & Jones, 2008). Japanese folk-medicine has utilized resveratrol in the treatment of inflammatory diseases for many years (Kubo, et. al, 1981). Resveratrol has the potential to control muscle wasting and fatigue by: 1.) decreasing or reversing tumor growth, and 2.) decreasing or reversing the inflammatory process.

Many studies have shown that resveratrol possesses anticancer properties in both animal models and in cultured human tumor cells by stimulating cancer cell death (Udenigwe, Ramprasath, Aluko, & Jones, 2008). By decreasing tumor size or increasing tumor apoptosis, resveratrol may decrease the amount of inflammatory cytokines released in response to tumor growth, and therefore, decrease muscle wasting and fatigue. Also, studies have shown that resveratrol blocks activation of NF-kB and decreases cytokine synthesis in response to stimulation of the inflammatory response (Wyke, Russell & Tisdale, 2004).

NF-kB activation is associated with tumor cell survival and increased muscle wasting (Baldwin, 2001). By interfering with the activation of NF-kB, resveratrol has the potential to decrease the activation of the ubiquitin proteosome pathway that leads to muscle degradation. Many studies have shown that resveratrol has the ability to

suppress TNF-α (Das & Das, 2007). By suppressing the release of TNF-α, resveratrol may inhibit release of NF-kB, and therefore inhibit activation of the ubiquitin-proteosome pathway, which leads to myofibrillar degradation. A study by Singletary et al. showed that grape juice concentrate delayed the onset of tumors in mice (2003-double check citation). Another study, by Wyke, Russell, and Tisdale, showed that resveratrol attenuated both weight loss and muscle protein degradation in tumor-bearing mice (2004). Thus, the question addressed by this study is: Would resveratrol supplements decrease or prevent muscle wasting and reduce fatigue in tumor-bearing mice compared to mice who are not given the supplement?



Methods

This study was a quantitative, experimental design using 28 age-matched adult C57BI/6 female mice which were divided into 4 groups: Tumor, Tumor + Resveratrol, Control, and Control + Resveratrol. Each mouse was housed individually, and acclimated to the environment for 10 days. One half of the mice were inoculated subcutaneously between the scapulae with 5x105 Lewis Lung Carcinoma tumor cells in

0.2 ml saline; controls were injected with 0.2 ml saline. Growth of the tumor between the scapulae does not impair the locomotor ability of the mice. One half the tumor-injected and one half the saline injected animals were implanted with a pellet designed to release 1 mg/kg/day resveratrol, over 21 days, and one half received a placebo pellet of the same size. Mice were weighed and food and water intake were measured on day 0, 7, 14, and 19.

Measurement of muscle wasting

For each mouse, the two gastrocnemius muscle were dissected and weighed at the time of sacrifice. Muscle weight was calculated as the average of the two muscles. Because muscle mass is expected to be less in smaller animals, relative gastrocnemius mass was determined by dividing average muscle weight by body weight.

Measurement of inflammation

Systemic inflammation in the mice was determined by dissecting and measuring spleen weight at the time of sacrifice. With increased inflammatory activity, the spleen increases in size and weight.

Measurement of Fatigue

Fatigue was modeled as a decline in the voluntary wheel running activity (VWRA) of the mice. Healthy mice can run up to 2 miles during the night. As muscle wasting proceeds, the mice run for less amount of time. VWRA was measured using a system triggered by a magnet embedded in the wheel that tripped a switch connected to a

signal processor and connected to a computer programmed to continuously record the total number of wheel turns in a preset period of time. VWRA was measured at days 0, 7, 14, and 19 of tumor growth. To control for individual variations in VWRA, the change in VWRA from days 7 to 14, and days 14-19 was also calculated.

Measure of Biomarkers

MAFbx and MURF1 are biomarkers that indicate muscle wasting. To measure these biomarkers, total RNA was extracted from 100 mg of frozen gastrocnemius muscle in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 1 µg RNA was treated with DNase 1 (Invitrogen) and reverse transcribed to cDNA using the Iscipt cDNA synthesis kit (BioRad). Real time PCR was performed using primer pairs for MAFbx and MURF1. All reactions were performed in duplicate using 25 ng of cDNA in a final reaction volume of 25 µl using the iCycler iQ5 (BioRad). The reaction conditions were 95 °C for 15 s and 60 °C for 1 min for 40 cycles after the initial denature at 95 °C for 10 min. The results for MAFbx and MURF1 were normalized to GAPDH and expressed as 2 CT (normalized expression ratio).

Statistical Analysis

Data was analyzed using two way (tumor/drug) analysis of variance. Bivariate correlations between the dependent variables were also analyzed using SPSS version 16.

Results

As shown in Figure 1, tumor had a significant effect on muscle mass ($p < 0.001$). Resveratrol had no effect on gastrocnemius muscle mass ($p=0.38$). These findings suggest that this dose of resveratrol did not affect the mechanisms responsible for tumor-induced muscle wasting. However, resveratrol had a different effect on muscle mass in tumor bearing and control mice ($p = 0.03$).

As shown in Figure 2, tumor had a significant effect on spleen weight ($p=0.005$). This reflects an increased inflammatory response induced by the tumor. Resveratrol had no effect on spleen weight ($p=0.88$).

Neither tumor growth or resveratrol had a significant effect on VWRA. However, when day 19 wheel counts were subtracted from day 14 wheel counts to produce a measure of change in VWRA, there was a significant effect of tumor growth on VWRA ($p=0.009$), but not resveratrol. Tumor mice VWRA declined by 182 turns, while control mice VWRA increased by 752 turns.

As shown in table 1, there was a significant negative correlation between spleen weight and relative gastrocnemius weight ($r= -0.68$) and between spleen weight and VWRA ($r= -0.67$), supporting the premise that systemic inflammatory response (increased spleen weight) contributes to skeletal muscle wasting (decreased muscle mass), and fatigue (decreased VWRA). There was also a significant positive correlation between muscle mass and VWRA, supporting the premise that muscle wasting contributes to fatigue.

As shown in figures 3 and 4, expression of both MAFbx and MURF1 mRNA were increased in gastrocnemius muscles of tumor-bearing mice. This would explain in part

the smaller muscle mass in the tumor-bearing mice. Additionally, as shown in table 1, expression of these biomarkers of myosin degradation were positively correlated with spleen weight, and negatively correlated with relative gastrocnemius weight, supporting the idea that inflammation plays a key role in tumor-induced muscle wasting.

Figure 1

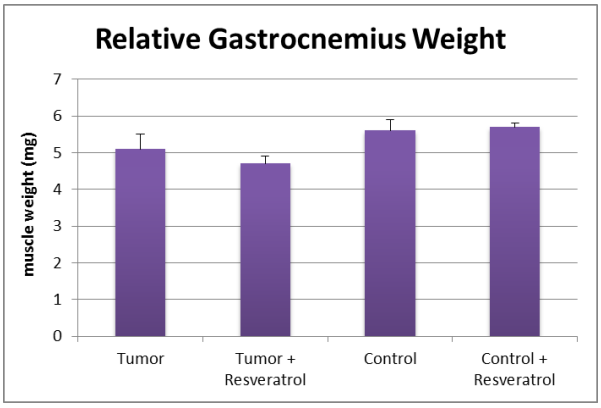
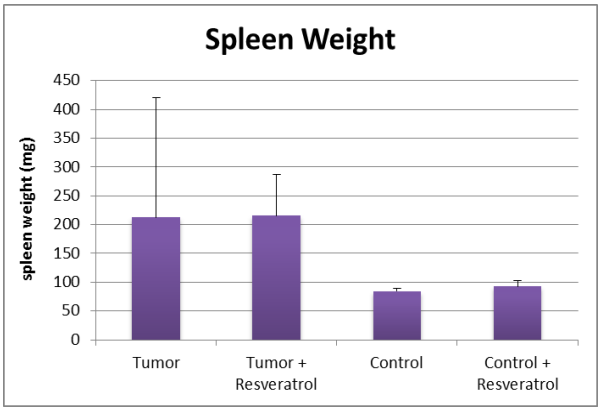


Figure 2



Bivariate Correlations, p < 0.05					
	Relative Gastro	Spleen Weight	VWRA Day 19	MAFbx	MURF1
Relative Gastroc	1	-0.68	0.58	-0.56	-0.71
Spleen Weight		1	-0.5	0.90	0.71
VWRA Day 19			1	-0.31	-0.20
MAFbx				1	0.84
MURF1					1

Figure 3

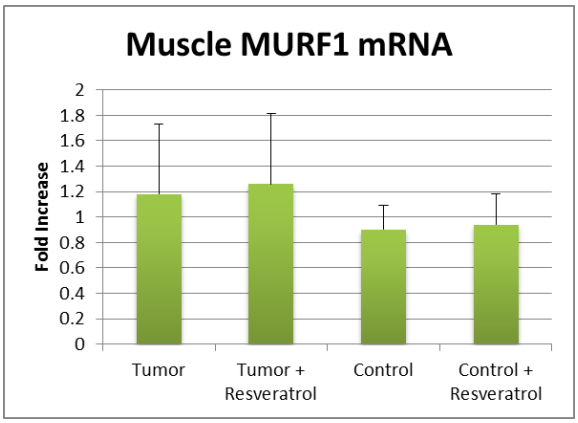
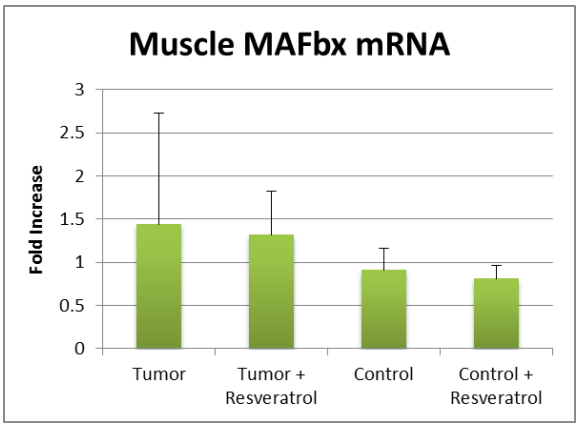


Figure 4



Conclusion

Cancer cachexia affects more than half of all cancer patients, causing weakness and fatigue, which contributes to worse outcomes and decreased quality of life. Although there is no specified cause of cachexia, studies suggest that an increased inflammatory process contributes to the disease process. Our study revealed that tumor bearing mice developed enlarged spleens over the course of the project, suggesting that the inflammatory response is triggered by the presence of tumor. Moreover, the tumor-bearing mice had smaller muscle mass, indicative of skeletal muscle wasting associated with cancer cachexia. Though resveratrol has been shown to have anti-inflammatory effects in previous studies, we found that resveratrol at 1

mg/kg/day did not have an effect on spleen size or muscle wasting in our mouse model of cancer cachexia.

In mice, fatigue is modeled as reduced voluntary wheel running. In the present study, there was a wide range of VWRA between mice, but no significant group differences in VWRA were detected. However, when day 19 wheel counts were subtracted from day 14 wheel counts to produce a measure of change in VWRA, VWRA was significantly less in the tumor-bearing mice when compared to the control mice.

Furthermore, VWRA was significantly correlated with spleen size and muscle mass, supporting an association between the inflammatory response in tumor-induced muscle wasting and fatigue. The collective data suggests that repeating this study using increased doses of resveratrol may be useful in determining whether the anti-inflammatory actions of resveratrol on cancer cachexia as demonstrated in previous studies have an effect. Further studies are needed to explore the cause of cancer cachexia and interventions that may counteract it.

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